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Anticancer Therapeutics That Target Selenoenzymes: Synthesis, Characterization, in vitro Cytotoxicity, and Thioredoxin Reductase Inhibition of a Series of Gold(I) Complexes Containing Hydrophilic Phosphine Ligands

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Gold(I) complexes bearing water-soluble phosphine ligands, including 1,3,5-triaza-7-phosphaadamantane (PTA), 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane (DAPTA), and sodium triphenylphosphine trisulfonate (TPPTS), in combination with thionate ligands, were screened for their antiproliferative activities against human ovarian cancer cell lines A2780 either sensitive or resistant to cisplatin. In addition, the compounds were screened for their inhibition of mammalian thioredoxin reductases (TrxR), enzymes that are overexpressed in many tumor cells and contribute to drug resistance. The gold(I)–phosphine complexes efficiently inhibited cytosolic and mitochondrial TrxRs at concentrations that did not affect the related oxidoreductase glutathione reductase (GR). Additional complementary information on the enzyme metallation process and potential gold binding sites was obtained through the application of a specific biochemical assay using a thiol-tagging reagent, BIAM (biotin-conjugated iodoacetamide).

Introduction

Following the clinical success of cisplatin, many platinum and non-platinum metallodrugs have been, and are currently being, investigated as experimental antitumor agents.[1–8] In particular, gold-based compounds are promising anticancer drugs, and a significant number of gold(III) and gold(I) compounds, with highly different chemical structures, have been shown to manifest outstanding antiproliferative activities against various human cancer cell lines.[9,10,11] The clinically established anti-arthritic gold(I) species, auranofin (1-thio-β-D-glucopyranosato)(triethylphosphine)gold 2,3,4,6-tetraacetate (Figure 1), and various other gold–phosphine compounds show significant antitumor properties, both in vitro and in vivo.[12,13] Much effort has been directed toward understanding the mode of action of this class of cytotoxic agents and their preferred protein targets, as it has become increasingly evident that DNA is not the unique target for these compounds.[7,14,15]

One class of putative targets for anticancer gold-based drugs is the mammalian thioredoxin reductases (TrxRs; EC 1.8.1.9), large homodimeric proteins that play a crucial role in the intracellular redox balance.[16] Two major isoforms are known, a cytosolic (TrxR1) and a mitochondrial form (TrxR2), although their main function is the same: the reduction of a 12 kDa disulfide protein, thioredoxin (Trx), to its dithiolic form. The view that TrxRs are effective targets for inorganic compounds is supported by a recent mechanistic investigation of arsenic trioxide, a potent TrxR inhibitor now approved for promyelocytic leukemia treatment.[17] Inhibition of TrxR by gold compounds appears to be a common mechanistic characteristic that explains (albeit to varying extents) their cytotoxic action. It has also been shown that efficient inhibition of TrxRs may lead to apoptosis through a mitochondrial pathway.[18,19] The cysteine thiol, which functions in the active site, as well as the redox-active selenocysteine, are the proposed TrxR target residues for gold(I) complexes.[20]

Based on the findings mentioned above, we report herein the correlation between cytotoxicity and TrxR inhibitory properties of a series of gold(I) complexes containing phosphine ligands (Figure 1). Following previous research of water-soluble gold complexes,[21–23] the compounds described herein contain water-soluble phosphine ligands, including 1,3,5-triaza-7-phosphaadamantane (PTA), 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane (DAPTA), and sodium triphenyl-

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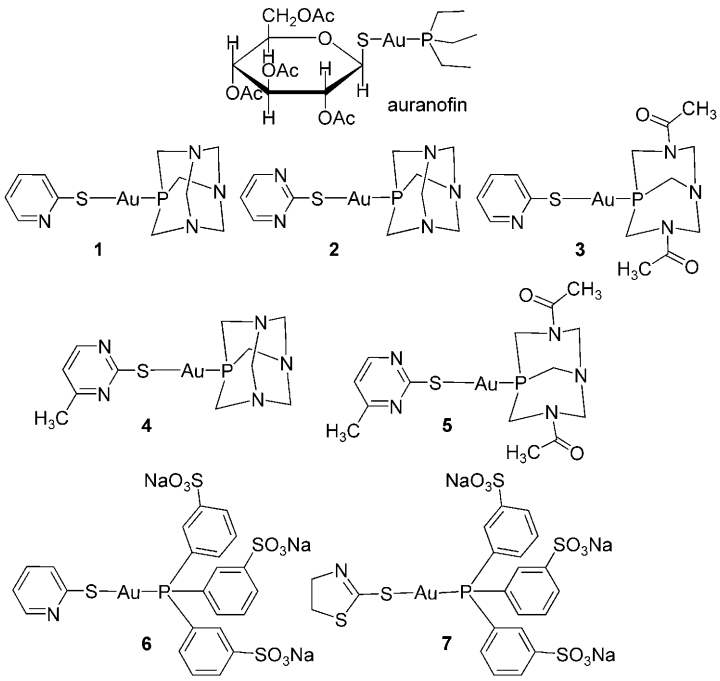

Figure 1. Structures of the gold(I) compound auranofin and compounds **1**–**7**.

phosphine trisulfonate (TPPTS), in combination with thionate ligands.[24,25] These compounds are not only water soluble, in contrast to related compounds with hydrophobic phosphines, but also exhibit excellent in vitro anticancer activity. Notably, several ruthenium complexes containing PTA ligands,[26–30] as well as osmium and rhodium derivatives,[31,32] have been shown to possess promising anticancer properties in recent years.[24,33] Overall, the results to date provide preliminary mechanistic insight for the series of gold(I) compounds under investigation.

Results and Discussion

Gold(I) compounds containing mono- or diphosphines have been shown to exhibit promising anticancer activity.[34,35] Nevertheless, one of the main limitations of these compounds is their poor water solubility, which can hinder bioavailability in vivo and lead to complicated formulation protocols. Based on a previous study describing compounds **1**–**5**,[24,25] which contain hydrophilic phosphine ligands, we decided to further investigate the biological properties of these complexes, together with two novel related complexes **6** and **7** (Figure 1). Complexes **6** and **7**, containing the TPPTS ligand in place of PTA and DAPTA, were prepared analogously to the previously described compounds, as reported in the experimental section. Compounds **1**–**7** are stable in aqueous solution over prolonged periods as evidenced by NMR spectroscopy and mass spectrometry (data not shown).

Biological evaluation of 1–7

The gold(I) compounds (**1**–**7**) were screened for cytotoxicity against the cisplatin-sensitive and cisplatin-resistant A2780 human ovarian cancer cell lines (Table 1). Dose-dependent inhibition of cell growth was observed in both cell lines with IC_{50} values ranging from 4 to 30 μM. Interestingly, higher cytotoxicity was observed in the resistant cell line, indicating that these complexes are able to overcome the resistance to cisplatin. Similar results were found with auranofin upon treating other ovarian cancer cells sensitive and resistant to cisplatin.[36] Resistance to cisplatin has been attributed to decreased uptake mediated by copper transporters[37] and increased levels of detoxification proteins, including metallothionines and glutathione transferase.[38,39] Thus, it would appear that the uptake and/or detoxification pathways of **1**–**7** differ significantly from those of cisplatin and also suggests that the primary biomolecular targets are not the same.

Table 1. IC_{50} values of **1**–**7** against human ovarian carcinoma cell lines sensitive (A2780/S) or resistant to cisplatin (A2780/R), as compared with cisplatin.

	Compound	IC_{50} [μM][a] A2780/S	A2780/R
	cisplatin	4.3 ± 0.5	18.2 ± 1
	auranofin	1.25 ± 0.5	1.5 ± 0.5
1	[Au(SPy)(PTA)]	9.6 ± 2.3	8.2 ± 2.8
2	[Au(SPyrim)(PTA)]	5.7 ± 1.9	4.1 ± 2.5
3	[Au(SPy)(DAPTA)]	12.1 ± 1.0	8.0 ± 1.6
4	[Au(SMePyrim)(PTA)]	7.8 ± 2.0	4.7 ± 1.7
5	[Au(SMePyrim)(DAPTA)]	19.5 ± 4.3	9.7 ± 3.0
6	[Au(SPy)(TPPTS)]	21.5 ± 1.5	13.8 ± 2.5
7	[Au(STz)(TPPTS)]	30.2 ± 1.2	16.2 ± 3.0

[a] Values are the mean ± SE of at least three determinations.

To evaluate the reactivity of the gold complexes with DNA, which is the main target of cisplatin,[2] the influence of **1**–**7** on plasmid DNA was monitored, following incubation, by agarose gel electrophoresis. Figure 2 shows a representative gel elec-

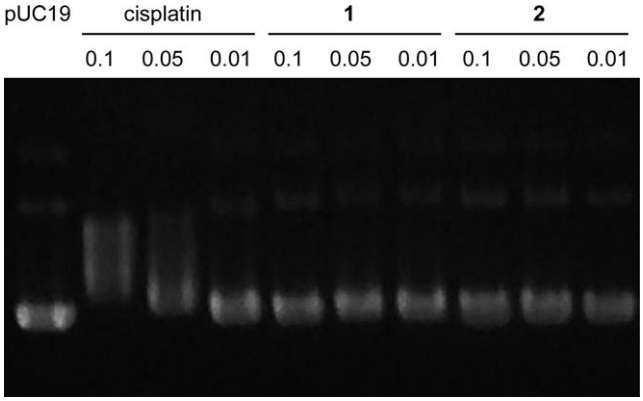

Figure 2. Gel electrophoresis of pUC19 plasmid DNA treated with various concentrations of cisplatin, **1**, and **2** [$r = 0.1$, 0.05, and 0.01; r = (metal complex):(DNA base pairs)] after 24 h incubation at 37 °C.

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trophoresis separation of plasmid pUC19 DNA after 24 h incubation at 37 °C with complexes **1**, **2** and cisplatin. Cisplatin has a marked effect on the DNA, inducing unwinding, whereas the gold complexes do not appear to alter plasmid DNA mobility, indicating that DNA is not a relevant target for these complexes.

Because TrxR is a potential target,[19] in vitro inhibition of rat TrxR by **1**–**7** was studied using an established protocol (Figure 3 and Table 2), with each compound inhibiting both TrxR1 and TrxR2. The IC_{50} values of TrxR inhibition for **1**–**7** are all in the low nanomolar range; the inhibitory effect is approximately fourfold higher for TrxR1 than for TrxR2. There are no large variations in the inhibitory effects of the gold compounds, with IC_{50} values in the range of 0.6–1.9 nM for TrxR1 and 4–6.9 nM for TrxR2. The most effective inhibitor of TrxR1 is [Au(SPy)TPPTS] **6**, whereas [Au(SMePyrim)PTA] **4** demonstrates the highest inhibitory activity toward TrxR2; both have IC_{50} values similar to that of auranofin. This differential inhibitory effect between these cytosolic and mitochondrial thioredoxin reductases was previously observed with other gold complexes,[19] ruthenium compounds[40] and calcium ions.[41] Interestingly, both ruthenium compounds and calcium ions strongly inhibit the cytosolic isoform but have only a minor effect on the mitochondrial isoform. These differences are attributed to the sequence variations between the two isoforms, with the TrxR1 active site being more acidic than that of TrxR2,[42] explaining the lower sensitivity of the latter enzyme to calcium ions.

Table 2. IC_{50} values for TrxR and GR inhibition calculated from the data shown in Figures 3 and 4.

	Compound	IC_{50} [nM] TrxR1	TrxR2	GR
	auranofin	0.74 ± 0.20	2.45 ± 0.32	> 50 μM
1	[Au(SPy)(PTA)]	1.61 ± 0.21	4.12 ± 0.28	1970 ± 20
2	[Au(SPyrim)(PTA)]	1.92 ± 0.15	6.73 ± 0.25	2176 ± 15
3	[Au(SPy)(DAPTA)]	1.54 ± 0.17	6.20 ± 0.35	2833 ± 36
4	[Au(SMePyrim)(PTA)]	0.96 ± 0.08	4.06 ± 0.41	1455 ± 35
5	[Au(SMePyrim)(DAPTA)]	1.55 ± 0.16	5.86 ± 0.32	3598 ± 18
6	[Au(SPy)(TPPTS)]	0.62 ± 0.04	6.95 ± 0.14	2989 ± 45
7	[Au(STz)(TPPTS)]	1.67 ± 0.05	6.82 ± 0.25	ND[a]

[a] Not determined.

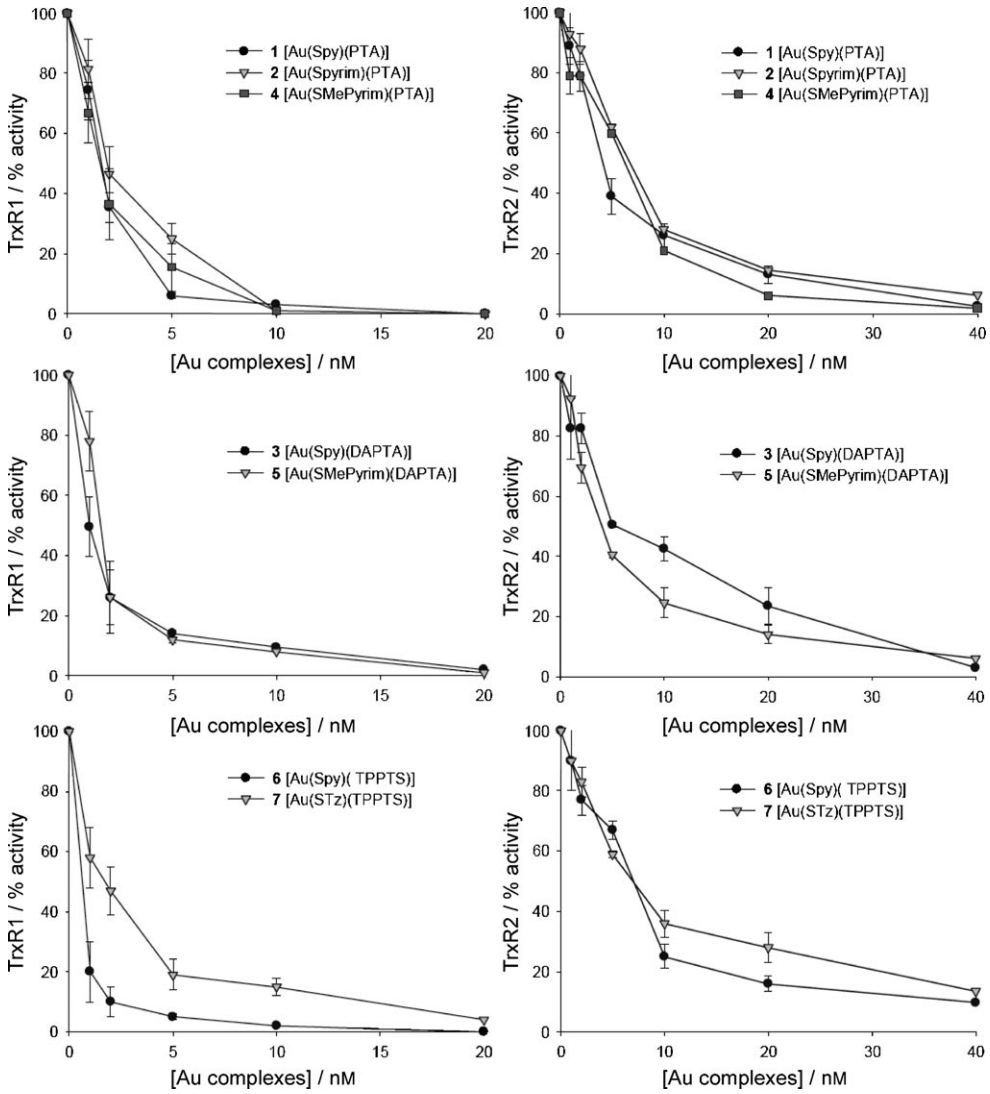

Figure 3. Effect of **1**–**7** on cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductase activity. The various gold complexes, at the reported concentrations, are indicated by each set of curves.

Complexes **1**–**7** were also screened for inhibition of glutathione reductase (GR), a pyridine disulfide oxidoreductase able to maintain glutathione in its reduced state. The gold complexes were able to inhibit this enzyme (Figure 4), albeit at much higher concentrations than those observed for TrxR inhibition. In fact, TrxRs, in contrast to GR, are endowed with a thiol-selenol C-terminal residue that appears to be a sensitive target of gold complexes, which bind to this residue with high affinity.[19] Overall, **4** appears to possess the optimum chemical features to inhibit all three enzymes.

The mechanism of enzyme inhibition by gold complexes has been partially investigated. It has been established that, in order to afford effective TrxR inhibition, pre-reduction of the enzyme by NADPH is essential, in order to convert the active site cysteine/selenocysteine residues to the S(Se)H "gold-reactive" form. Figure 5 compares

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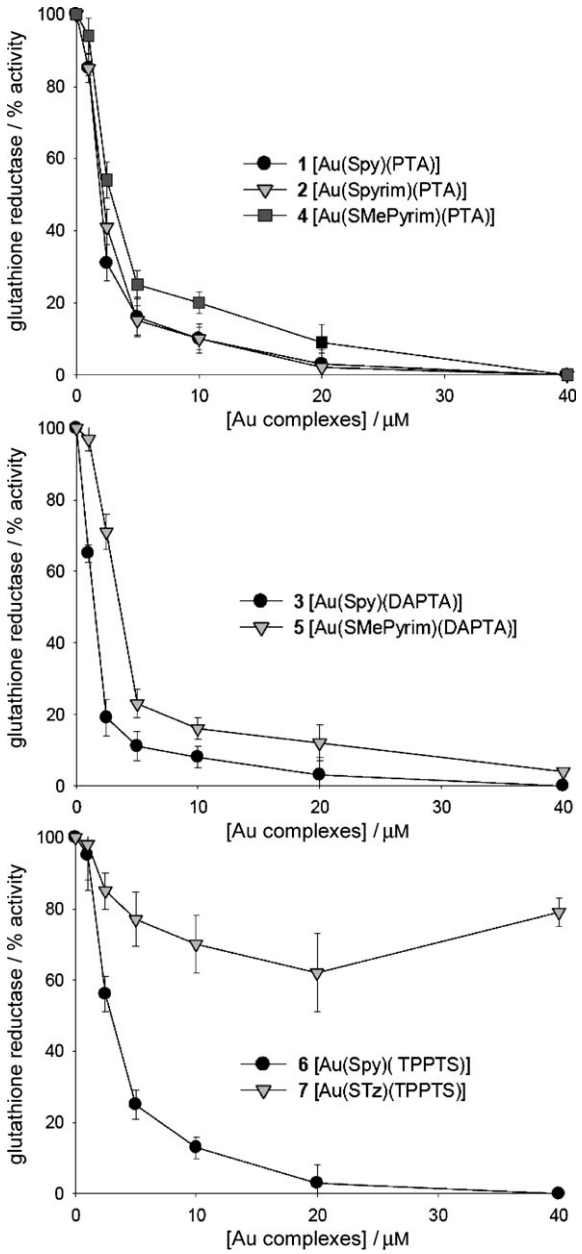

Figure 4. Effect of **1**–**7** on glutathione reductase (GR) activity. The various gold complexes, at the reported concentrations, are indicated by each set of curves.

the effect of the benchmark gold complex auranofin to [Au(SPyrim)(PTA)] **2**, with respect to TrxR1 inhibition under reducing (+NADPH) and oxidizing (−NADPH) conditions. Following gel-exclusion chromatography to eliminate the excess reagent, auranofin and compound **2** in the absence of NADPH were found to be scarcely bound. In contrast, following pre-reduction of the enzyme with NADPH, both gold complexes inhibited enzyme activity almost completely. These results indicate that the thiol/selenol motif at the C-terminal residue of the enzyme is the preferential site of inhibition by gold complexes.

Additional complementary information regarding the enzyme metallation process and possible binding sites was obtained through the application of a specific biochemical assay

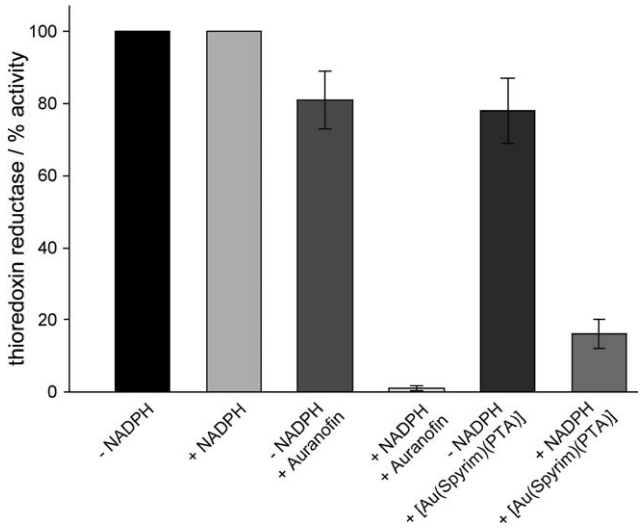

Figure 5. Effect of differing redox conditions of thioredoxin reductase on inhibitory effects exerted by gold(I) complexes. Cytosolic thioredoxin reductase activity was observed in the presence of NADPH (reduced conditions) or in its absence (oxidized conditions), together with 1 μM auranofin or [Au(SPyrim)(PTA)] **2**. Enzyme activity was estimated using the DTNB method following column filtration.

that relies on the thiol-tagging reagent, BIAM (biotin-conjugated iodoacetamide).[43] BIAM selectively alkylates TrxR in a pH-dependent manner; at pH 6.0 only selenocysteines and low-pK_a cysteines are alkylated, whereas at pH 8.5, all cysteines and selenocysteines are modified. In our experiments, TrxR1 was treated with each metal complex in turn, after which sample aliquots were treated with BIAM, either at pH 6.0 or 8.5, and the mixtures analyzed by SDS-PAGE. BIAM-labeled proteins were detected with horseradish peroxidase-conjugated streptavidin (see Figure 6 and Experimental Section for further details). The immunoblotting indicated that all of these gold

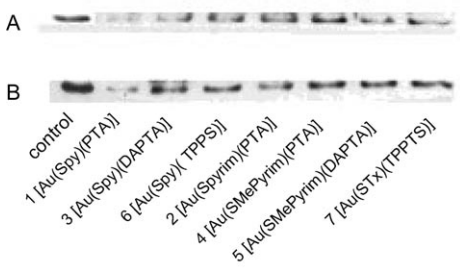

Figure 6. Alkylation of TrxR1 with BIAM following treatment with gold(I) complexes. Thioredoxin reductase was incubated as indicated in the Experimental Section at A) pH 6.0 or B) pH 8.2.

complexes are able to target, although to slightly varying extents, the cysteine and selenocysteine residues present in the redox-active motif.

Notably, previous studies on the interactions of gold(I) complexes, including the auranofin derivative [$AuCl(PEt_3)$], with proteins provided evidence for gold binding to cysteine as well as histidine residues.[19,44] Moreover, investigation of the

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reactions of auranofin with serum albumin showed that, upon gold binding to Cys34, the thioglucose ligand was displaced with the phosphine moiety remaining attached.[45] A similar mechanism of thiolate release may be responsible for the activity of the series of gold(I) compounds described here. Further studies are underway to establish whether TrxR histidines could also form covalent interactions with gold ions and to determine the nature of the protein-bound metallofragments.

Conclusions

A series of water-soluble gold(I) compounds were evaluated for their cytotoxic properties against both cisplatin-sensitive and -resistant human A2780 ovarian cancer cell lines. All compounds showed promising antiproliferative effects, particularly against the cisplatin-resistant cell line, with IC_{50} values in the 4–16 μM range. In order to investigate the possible mechanism of action of this family of gold(I) complexes, further studies were undertaken. First, it was possible to exclude DNA as a relevant target, because no reactions between the complexes and DNA were observed. However, the compounds were found to inhibit cytosolic and mitochondrial thioredoxin reductases in vitro. Indeed, the central functions of the thioredoxin system make it an attractive target for antitumor drug development,[46] particularly for anticancer gold-based compounds. Overall, the reported compounds were excellent inhibitors of both TrxR1 and TrxR2 (with IC_{50} values in the low nanomolar range). Complexes **4** [Au(SMePyrim)(PTA)] and **6** [Au(SPy)(TPPTS)] were among the most effective compounds.

Studies also indicated that the selenol in the active site of TrxRs is the preferential gold(I) binding site. This hypothesis was further confirmed by the comparatively poor inhibition of GR, as mammalian TrxRs contain a selenocysteine residue at the C-terminal active site that is crucial for catalysis and is not found in GR.[47] This redox center is located on a flexible arm, is solvent-exposed, and is reactive toward electrophilic agents.[48] Pre-reduction of TrxRs by NADPH was determined necessary for TrxR inhibition by the complexes, as binding of NADPH to the TrxR orients the C terminus toward the enzyme surface,[49] allowing the selenol group to bind gold atoms. The BIAM assay confirmed that a number of cysteines, as well as the active site selenocysteine, are modified by gold compounds. Overall, these results are very encouraging, indicating that these complexes behave as very selective TrxR inhibitors. Moreover, the fact that these complexes are stable and can be administered in aqueous solutions provides additional advantages over other compounds described in the literature.

Experimental Section

General methods and materials

PTA,[50] DAPTA,[51] [AuCl(PTA)],[52] [AuCl(TPPTS)],[53] [AuCl(DAPTA)],[25] [Au(SPy)(PTA)] (**1**), [Au(SPyrim)(PTA)] (**2**), [Au(SPy)(DAPTA)] (**3**), [Au(SMePyrim)(PTA)] (**4**) and [Au(SMePyrim)(DAPTA)] (**5**) were prepared according to literature procedures.[24,25]pUC19 plasmid DNA was purchased from Sigma. ^{1}H, ^{13}C, and $^{31}P\{^{1}H\}$ NMR spectra NMR spectra were recorded using 400 MHz Varian INOVA or 400 or 500 MHz Bruker Avance spectrometers. Chemical shifts (δ) are quoted in parts per million relative to external TMS (^{1}H, ^{13}C) or 85% H_3PO_4 (^{31}P) and coupling constants (*J*) are reported in Hertz. FAB mass spectra were measured using a Micromass Autospec spectrometer in positive-ion mode with *m*-nitrobenzyl alcohol (NBA) as the matrix. IR spectra were recorded as KBr or polyethylene disks using a Nicolet Impact 410 FTIR or JASCO FTIR (far-IR) spectrometers. Elemental analyses were obtained in-house by using a LECO CHNS-932 microanalyzer.

General synthesis of [Au(SR)(TPPTS)] complexes 6 and 7

[AuCl(TPPTS)] (0.206 g, 0.257 mmol) was added to a solution of KOH (0.022 g, 0.385 mmol) in MeOH (~10 mL) containing 2-pyridinethione (HSPy) for **6**, or 2-thiazoline-2-thiol (HSTz) for **7** (308 mmol). After stirring for ~20 h, the solution was evaporated to dryness in vacuo, and the residue extracted with CH_2Cl_2 (3× 10 mL). The combined extracts were passed through Celite and concentrated in vacuo to ~5 mL. Addition of pentane or Et_2O was used to precipitate the products, which were isolated by filtration and air-dried.

Spectroscopic data for [Au(SPy)(TPPTS)] (6): Yield: 78%, light yellow solid. Water solubility: 7.7 g L^{-1}; ^{1}H NMR (400 MHz, D_2O, 25 °C): $\delta = 6.96$ (dt, $J = 5.3$, 1.3 Hz, 1H, py-H^4), 7.42 (dt, $J = 7.9$, 1.9 Hz, 1H, py-H^5), 7.55–7.64 (m, 6H, *o*-$PhSO_3Na$ (H^6), *m*-$PhSO_3Na$), 7.87–7.94 (*m*, 6H, *o*-$PhSO_3Na$ (H^2), *p*-$PhSO_3Na$), 7.63 (d, $J = 8.0$ Hz, 1H, py-H^6), 8.13 ppm (dd, $J = 4.7$, 1.0 Hz, 1H, py-H^3); $^{31}P\{^{1}H\}$ NMR (D_2O, 25 °C): $\delta = 37.65$ ppm; FAB MS: $m/z = 876$ $[M]^+$; $C_{23}H_{16}AuNNa_3O_9PS_4$ (875.5): calcd: C 31.55, H 1.84, N 1.60, found: C 31.87, H 2.23, N 1.31.

Spectroscopic data for [Au(STz)(TPPTS)] (7): Yield: 63%, light yellow solid. Water solubility: 2.9 g L^{-1}. ^{1}H NMR (400 MHz, D_2O, 25 °C): $\delta = 3.30$ (t, $J = 8.1$ Hz, 2H, thiazoline), 4.09 (t, $J = 8.1$ Hz, 2H, thiazoline), 7.58–7.76 (m, 6H, *o*-$PhSO_3Na$ (H^6), *m*-$PhSO_3Na$), 7.94–8.02 ppm (*m*, 6H, *o*-$PhSO_3Na$ (H^2), *p*-$PhSO_3Na$); $^{31}P\{^{1}H\}$ NMR (162 MHz, D_2O, 25 °C): $\delta = 36.95$ ppm; FAB MS: $m/z = 883$ $[M]^+$; $C_{21}H_{16}AuNNa_3O_9PS_5$ (883.59): calcd: C 28.55, H 1.83, N 1.59, found: C 28.78, H 1.87, N 1.36.

Cells and cell treatment

Human A2780 and A2780cisR cells were obtained from the European Centre of Cell Cultures (ECACC, Salisbury, UK). All cell culture reagents were obtained from Gibco-BRL (Basel, Switzerland). Cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics. Stock solutions of the complexes (16 mM in DMSO) were diluted in complete medium to the required concentration. DMSO at similar concentrations did not show any effects on cytotoxicity.

Determination of cytotoxicity

Cells were grown in 96-well cell culture plates (Corning, NY, USA) at a density of 25×10^3 cells per well. The culture medium was replaced with fresh medium containing complexes **1**–**7** at concentrations varying from 0 to 20 μM, with an exposure time of 72 h. Thereafter, the medium was replaced by fresh medium, and cell survival was measured using the MTT test as previously described.[54] Briefly, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT, Merck) was added at 250 μg mL^{-1}, and incubation was continued for 2 h. The cell culture supernatants were

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then removed, the cell layer was dissolved in DMSO, and absorbance at 540 nm was measured in a 96-well multi-well plate reader (iEMS Reader MF, Labsystems, Bioconcept, Switzerland) for comparison with the values of control cells incubated in the absence of complexes. Experiments were conducted in quadruplicate wells and repeated at least twice.

Interactions with DNA

Adducts with pUC19 plasmid DNA were prepared by adding the required volume of a freshly prepared solution of metal complexes in MilliQ water. The concentration of pUC19 DNA in the reaction mixture was 75 ngL^{-1}, while the concentration of the complexes was varied to produce different metal-to-base-pair stoichiometries (0.1, 0.05 and 0.01). The mobility of the metal-complex-treated pUC19 samples was analyzed by gel electrophoresis on a 0.8% (*w/v*) agarose gel (Boehringer-Mannheim, Mannheim, Germany) at 90 $V\,cm^{-1}$ at 25 °C for 6 h in Tris-acetate/EDTA buffer. The gel was stained for 30 min in 0.5 $g\,mL^{-1}$ (*w/v*) ethidium bromide, and the bands were analyzed with a UVP gel scanner.

Preparation of TrxR and GR

Highly purified cytosolic thioredoxin reductase (TrxR1) was prepared according to Luthman and Holmgren, starting from rat liver.[55] Mitochondrial thioredoxin reductase (TrxR2) was purified from isolated liver mitochondria following the procedure of Rigobello et al.[56] Proteins of the purified TrxR isoforms were assayed using the procedure of Lowry et al.[57] Yeast glutathione reductase was obtained from Sigma–Aldrich (St. Louis MO, USA).

Enzyme activity estimation

Thioredoxin reductase activity was determined by measuring the ability of the enzyme to directly reduce DTNB in the presence of NADPH.[55] Aliquots of highly purified TrxR1 (60 nM) and TrxR2 (130 nM) were pre-incubated for 5 min in 0.2 M Na/K phosphate buffer (pH 7.4), 5 mM EDTA and 0.25 mM NADPH, and in the presence of the various gold complexes (final volume 1 mL). At the end of the incubation time, the reaction was started with 1 mM DTNB and monitored spectrophotometrically at 412 nm for approximately 10 min. Glutathione reductase activity was measured after pre-incubation for 5 min in a solution containing the various gold complexes in 0.2 M Tris HCl buffer (pH 8.1), 1 mM EDTA, and 250 μM NADPH. The assay was initiated by the addition of 1 mM GSSG and followed spectrophotometrically at 340 nm.

Gel filtration

Thioredoxin reductase was incubated in 0.2 M Na/K phosphate buffer (pH 7.4), 0.2 mM EDTA and 0.4 mM NADPH for a final volume of 30 μL. After 2 min incubation the gold complexes (1 μM) were added, and the reaction continued for a further 10 min. Samples were then diluted by the addition of 30 μL incubation buffer, applied to a desalting column (Micro Bio-Spin, Bio-Rad), and centrifuged at 1000 *g* for 5 min. The filtration procedure was then repeated in order to remove any remaining traces of the gold complexes. Thioredoxin reductase activity was estimated using the DTNB method for the final filtrate as described above.

BIAM assay

TrxR1 (0.120 $mg\,mL^{-1}$) was first reduced with 200 μM NADPH in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. After 2 min, the gold complexes (20 μM) were added to achieve a final volume of 15 μL, and the reaction continued for 1 h at room temperature. Aliquots of 6 μL of each sample were treated with 100 μM BIAM for a final volume of 20 μL in 0.2 M HEPES-Tris, at either pH 6.0 or pH 8.5, for 30 min at 37 °C. The reaction was quenched by the addition of 10 μL loading buffer containing 0.1 M DTT, followed by heating at 100 °C for 10 min. Samples were then subjected to SDS-PAGE (7.5%) and subsequently transferred to a nitrocellulose membrane. The biotinyl carboxamidomethyl-labeled proteins were detected by horseradish peroxidase (HRP)-conjugated streptavidin followed by ECL.

Abbreviations

BIAM = biotin-conjugated iodoacetamide; DAPTA = 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane; ECL = enhanced chemiluminescence; GR = glutathione reductase; GSSG = oxidized glutathione; PTA = 1,3,5-triaza-7-phosphaadamantane; SPy = 2-pyridinethione; SPyrim = 2-thiopyrimidine; SMePyrim = 4-methyl-2-thiopyrimidine; STz = 2-thiazoline-2-thiol or S-thiazoline; Trx = thioredoxin; TrxR = thioredoxin reductase; TrxR1 = cytosolic thioredoxin reductase; TrxR2 = mitochondrial thioredoxin reductase; TPPTS = sodium triphenylphosphine trisulfonate

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